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Optimization of enzymatic synthesis of eugenol ester using statistical approaches

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ABSTRACT

Eugenol caprylate was synthesized using Lipozyme TLIM as the biocatalyst for the reaction. A two level Plackett–Burman (PB) experimental design was used for finding the significant reaction parameters. Response surface methodology (RSM) with a three-factor-five-level central composite rotatable design (CCRD) was further employed to study and optimize the reaction conditions. A good correlation between the predicted and actual responses showed that the generated model could adequately predict the conversion yield. The maximum conversion yield (72.2%) was obtained at the optimal condition of 65 °C, 250 rpm, 259 min, 100 mg enzyme, and 2:1 M ratio of eugenol/caprylic acid in solvent-free system.

1. Introduction

Aromatic essential oils are volatile liquids extracted from various plants that generally grow in tropical countries. They not only have pleasant fragrances, but also possess many useful biological activities (Bakkali et al., 2008). Eugenol (4-allyl-2-methoxyphenol) is the major constituent of several important essential oils such as clove, pimento berry, bay, nutmeg and cinnamon oil. It is commonly used as a fragrance and flavoring agent in a variety of cosmetics, and food products. Eugenol has shown antimicrobial, antioxidant, anti-inflammatory, antispasmodic, antidepressant, antigenotoxic, and anticarcinogenic properties (Awasthi et al., 2008; Sadeghian et al., 2008).

Besides their sweet aroma, esters of eugenol such as eugenol palmitate, eugenol myristate, and eugenol benzoate have been introduced as potential future drugs against many diseases (Awasthi et al., 2008; Sadeghian et al., 2008). Generally, esterification improves specific properties of the substrates such as emulsification and dispersion and overall quality of the consumer products (Salimon and Salih, 2009). Eugenol esters are usually produced by chemical esterification of the eugenol with acid chlorides in either aqueous solution of sodium 4-allyl-2-methoxyphenolate or hydrochloride salts of pyridine carboxyl chloride at 130 °C (Awasthi et al., 2008; Sadeghian et al., 2008). Compared to the classical method of esterification, enzymatic synthesis offers favorable advantages such as milder reaction conditions, low energy requirement, high yields and purity, shorter reaction time, and biocatalyst reusability (Freitas et al., 2010). Enzymatic synthesis of eugenol benzoate by a non-commercial immobilized *Staphylococcus aureus* lipase using chloroform as the solvent has been reported previously (Horchani et al., 2010). The maximum conversion yield of ester, obtained by optimization of four reaction parameters including temperature, enzyme amount, substrate molar ratio and solvent volume, was 75%.

In biotechnological production processes, optimization plays a significant role in the commercial success of the industry on the basis of quality, cost, and the process performance. The conventional method of optimization requires screening of large number of variables, conducting a large number of experiments, and involvement of lots of time and resources. Experimental design approaches provide an efficient way for the screening of main parameters from a large number of process variables (Deshmukh and Puranik, 2010). Among various designs that have been frequently used for screening process variables, the Plackett–Burman (PB) design has been found to be best suited for choosing the important parameters and obtaining the most information (Rao and Divakar, 2001). PB design is quite useful in preliminary studies for selecting variables that can be kept constant or

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eliminated prior to process optimization (Reddy et al., 2008). In addition, response surface methodology (RSM) as a fast and economical statistical technique can be used to determine the optimal conditions of a multivariable system. RSM has been extensively applied for optimization of enzymatic processes (Lee et al., 2010; Jeong et al., 2009).

In the present work, eugenol caprylate was synthesized using commercial immobilized *Thermomyces lanuginose* lipase, Lipozyme TLIM. The PB design was used to screen the significant variables, and RSM was further used to optimize the levels of the screened variables. The product was purified and phase diagrams of surfactant/water/eugenol ester ternary systems were also developed.

2. Materials and methods

2.1. Materials

Lipozyme® TLIM (commercial lipase from *Thermomyces lanuginose* immobilized on silica gel, activity of 170 IUN/g) was purchased from NOVO Nordisk A/S (Bagsvaerd, Denmark). Eugenol (> 98% purity) and caprylic acid (> 98% purity) were purchased from Fluka. Molecular sieve (3 Å, bead, 4–8 mesh, Sigma-Aldrich, USA) was used as the water adsorbent. Triton® X100 (TX100) was from Sigma-Aldrich. Sodium dodecylsulfate, SDS (> 95%), was purchased from Wako Pure Chemical Industries, Ltd., USA. All other chemicals used were of analytical grade.

2.2. Lipase-catalyzed esterification

Eugenol caprylate was obtained by the esterification of eugenol with caprylic acid using immobilized lipase (Scheme 1). Lipozyme TLIM, a rather cheap commercial lipase, was selected as the biocatalyst after preliminarily screening test of three commercial immobilized lipases including Novozym 435, Lipozyme RMIM, and Lipozyme TLIM.

Eugnol and caprylic acid with various molar ratios (0.2 (0.2 mmol eugenol:1 mmol acid)-2 (2 mmol eugenol:1 mmol acid)) were mixed in a 50 ml flask. Various amounts of hexane (0–10 ml) were added as solvent. Then Lipozyme TLIM (20–400 mg) and molecular sieve (0–400 mg) were added. The reaction was carried out under reflux system on magnetic stirrer (C-MAG HS7) at different temperatures and time periods, as presented in Table 1.

2.3. Analysis and characterization

The reaction was terminated by adding 5 ml of ethanol:acetone (50:50 v/v). The enzyme was filtered off and the remaining free acid in the reaction mixture was determined by titration with 0.1 mol L^{-1} NaOH using the pH meter model EUTECH (titration end point of caprylic acid = 9.4). The amount of reacted acid was determined from the values obtained for the control (without enzyme) and test samples. The ester formed was expressed as equivalent to conversion of the acid (Lee et al., 2010). Formation of the ester was confirmed by Fourier Transform Infra-Red (FTIR) spectrophotometer (Perkin Elmer, model 1650) with absorption bands of C=O bond of ester at 1707.45 cm^{-1}, and C–O stretching vibrations at 1121–1256 cm^{-1}. Production of eugenol ester was also characterized by gas chromatography/mass spectroscopy (GC/MS) on an Agilent (model GC 7890A; model MS 5975C; Agilent Technologies Inc., Palo Alto, Ca) instrument with a HP-5 MS column (0.25 mm × 30 m, 0.25 μm). The carrier gas was helium, and the total gas flow rate was 20 ml min^{-1}. The injection mode was splitless and the injector temperature was set at 300 °C. The oven temperature was maintained at 180 °C for 1 min, elevated to 250 °C at a rate of 20 °C min^{-1} and held for 1 min. The spectra were scanned within the range *m/z* 40–300.

GC–MS analysis shows the presence of the ester at a retention time of 4.285 min. The mass spectrum of ester product showed a molecular ion peak at *m/z* 290 that corresponded to molecular formula of eugenol caprylate (C18H26O3). Cleavage of the ester gave the fragment peak at *m/z* 164 related to the eugenol part $[M+H]^+$. The fragment ion peak at *m/z* 149 is a result of formation of $[C_9H_9O_2]^-$. Other fragments ions were also observed at *m/z* 41, 57, 91, 103 and 131.

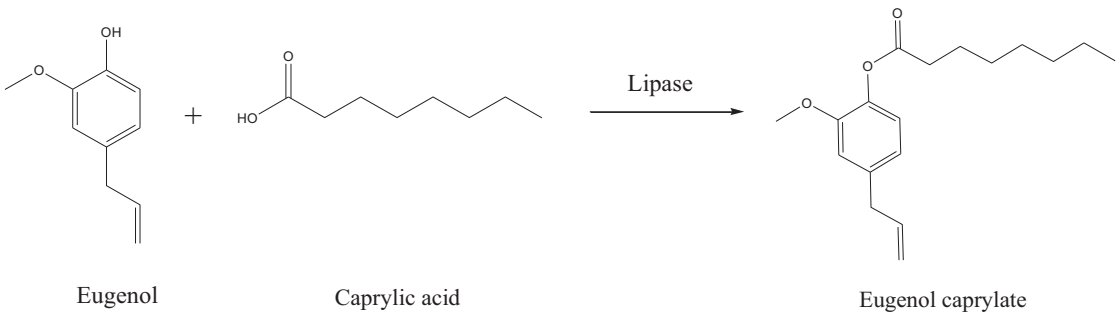
2.4. Screening of parameters by Plackett–Burman(PB) design

For the selection of significant parameters which were effective on the production of the ester, the Plackett–Burman design based on the first order model was employed (Eq. (1)).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i \quad (1)$$

where, Y is the estimated target function, β_0 and β_i are constant regression coefficients, X is independent variable and k is number of variables.

The PB design helps to avoid keeping the important factors at a random constant level. A set of 12 experiments was constructed for seven variables using the Design Expert Software, version 6.07 (State Ease Inc., Statistic made Easy Minneapolis, MN, USA), with each variable represented at two levels, high and low. The parameters which were considered to be important for ester production were temperature, time, eugenol:acid molar ratio, enzyme amount, molecular sieve amount, solvent volume, and mixing rate. The experimental design and actual level of the variables are shown in Table 1.



Scheme 1. Esterification of eugenol with caprylic acid.